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Purification, Resolution, and Interaction of the Glucosyltransferases of *Streptococcus mutans* 6715

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The extracellular glucosyltransferase produced by *Streptococcus mutans* 6715 was purified from culture supernatant fluids to a specific activity of 9.6 IU/mg of protein, with an overall recovery of 87%. The purified enzyme preparation, designated unfractionated enzyme (UFE), synthesized only water-insoluble glucans from sucrose during the initial stages of the reaction, although some water-soluble polymers accumulated after extended periods of incubation. It was free from measurable fructosyltransferase activity. The UFE preparation was resolved into two different catalytically active components by ethanol fractionation. One fraction (designated insoluble product enzyme [IPE]) synthesized water-insoluble glucans, whereas the other (designated soluble product enzyme [SPE]) produced primarily water-soluble glucans. The difference between the insoluble glucans made by the UFE preparation and those made by the IPE fraction appeared to be due to interaction of the SPE and IPE components in the UFE preparation. Addition of commercial dextrans or enzymatically prepared glucans to the glucosyltransferase assay altered the amounts of soluble and insoluble glucans synthesized by the UFE preparation. The molecular weights of the major enzymatically active proteins producing insoluble and soluble glucans were estimated by gel filtration chromatography to be 150,000 and 175,000, respectively.

The cariogenic potential of *Streptococcus mutans* appears to lie, in part at least, in its ability to synthesize extracellular and cell-bound glucosyltransferases that produce adhesive water-insoluble glucans of high molecular weight from sucrose (22, 24, 29, 31, 39). Adherence of the organism to smooth surfaces (e.g., teeth) appears to require the cell-bound enzymes (31, 32). Gibbons and Fitzgerald (20) have shown that *S. mutans* cells adhere to teeth coated with dextran and also agglutinate upon the addition of high-molecular-weight dextrans obtained from *Leuconostoc* strains. Dextranases (1, 14), antisera against glucosyltransferase preparations (17, 35), and various low-molecular-weight dextrans (21) interfere with adherence and/or agglutination.

Several distinct glucosyltransferases (dextransucrases) were isolated by Guggenheim and Newbrun (26) from culture supernatant fluids of *S. mutans* OMZ-176, a Bratthall serotype *d* strain (2). Analyses of the glucan products revealed differences in the relative amounts of α-1,3 and α-1,6 glucosidic linkages (4, 24). One of these enzyme preparations was found to make glucans containing 94% α-1,3 bonds (4). The extracellular glucan-synthesizing system of *S. mutans* HS-6 (serotype *a*) was resolved by Fukui and co-workers (15) into two enzyme fractions by utilizing agarose chromatography. The lower-molecular-weight fraction, purified to electrophoretic homogeneity, made water-soluble glucans with 94% α-1,6 glucosidic linkages. The high-molecular-weight fraction synthesized insoluble glucans, which were not further characterized. Working with culture supernatant fluids of strain HS-6, Mukasa and Slade (33) also found that both water-soluble and water-insoluble glucan-synthesizing fractions contained equal amounts of fructosyltransferase activity and reported that the preparation which made insoluble glucans existed as a high-molecular-weight complex. The glucosyltransferase activity of several other *S. mutans* strains, representing serotypes *a*, *b*, *c*, and *d* (16, 27), have also been separated into fractions catalyzing primarily soluble or insoluble glucan formation. Although Chludzinski et al. (6) did not characterize the glucan products formed by their enzyme preparations derived from strain 6715 (serotype *d*), they did find two separate activity bands on polyacrylamide gels after electrophoresis.

The present communication reports on the purification of the glucosyltransferase system of *S. mutans* 6715 (serotype *d*) and its resolution into two major enzyme components. The inter-

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action of these enzyme components and the effects of various glucans on the synthesis of polysaccharide products are discussed.

Preliminary reports of this work have been presented (10; J. E. Ciardi and C. L. Wittenberger, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P284, p. 188; J. E. Ciardi and C. L. Wittenberger, Fed. Proc. **33**:1435, 1974).

MATERIALS AND METHODS

Growth of *S. mutans*. *S. mutans* 6715-14 (14), obtained from R. McCabe (National Institute of Dental Research, Bethesda, Md.) was grown for 18 h at 37°C in a complex medium containing 0.5% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract, 0.5% K_2HPO_4, 0.05% Tween 80, and 0.5% mannitol, pH 7.6 (3). The final pH of the medium after growth was 5.7, and the absorbance of the cell suspension at 660 nm was approximately 1.9.

Enzyme assays. Glucosyltransferase activity was assayed by measuring the incorporation of the glucosyl moiety from [*U-glucosyl*-^{14}C]sucrose (New England Nuclear Corp., Boston, Mass.) into polysaccharides (37). The standard assay mixture contained enzyme, 100 mM imidazole hydrochloride buffer (pH 6.5), and 100 mM [*U-glucosyl*-^{14}C]sucrose (0.01 to 0.04 μCi/μmol of sucrose). The reaction mixture was incubated at 30°C and terminated at the appropriate time by placing it in a boiling-water bath for 1 to 2 min.

For total glucan determinations, 50- to 100-μl volumes of the reaction mixtures were transferred to Whatman 3MM filter disks (2.4 cm), washed once with 10 ml of absolute alcohol and four times with 5-ml volumes of 75% ethanol, and then counted in a liquid scintillation counter, using 5.0 ml of Aquasol (New England Nuclear Corp.). For quantification of water-soluble glucans, the reaction mixture was centrifuged at 35,000 × *g*, and 50- to 100-μl portions of the supernatant fluid were treated in the manner described for total glucan determination. The incorporation of radioactivity into water-insoluble glucans was measured on the 35,000 × *g* pellet after washing it twice with 10 mM imidazole hydrochloride (pH 6.5), dissolving it in 1 volume of 1 N NaOH, and transferring portions of the solution to Whatman 3MM filter disks. The filters were dried, washed twice with 25% ethanol (5 ml) and twice with distilled water (5 ml), and counted in Aquasol. The recovery of insoluble glucan, for the UFE preparation (see below), by this direct method was found to be between 94 and 102% of the value found by subtracting water-soluble glucans from total glucans. One glucosyltransferase unit is the amount of enzyme necessary to convert 1 μmol of glucose, derived from [*U-glucosyl*-^{14}C]sucrose, into radioactive glucans in 1 min at 30°C.

Where noted, the formation of water-insoluble glucans under the above-mentioned assay conditions (except for the use of unlabeled sucrose) was also measured turbidimetrically at 340 nm by a modification of the method described by Gibbons and Nygaard (22). After an initial lag period of approximately 10 min, the reaction proceeded at a linear rate, and this rate was used to measure insoluble glucan synthesis. For certain experiments, absorbance measurements at 340 nm were also used to estimate production of water-soluble glucan after the 35,000 × *g* supernatant fluid of the reaction mixture was treated with 3 volumes of absolute ethanol. Results obtained by the turbidimetric procedure were found to agree well with those carried out in parallel by the radioactive assay.

Fructosyltransferase was assayed exactly as described for glucosyltransferase, except that [*U-fructosyl*-^{14}C]sucrose (New England Nuclear Corp.) served as the substrate.

Thin-layer chromatography. Various radioactive glucans and other enzyme reaction products were also analyzed by thin-layer chromatography on silica gel F254 (E. Merck). Total reaction products were determined by this method in 20-μl portions of the reaction mixture, and water-soluble products were determined in 20-μl portions of the 35,000 × *g* supernatant solution of the reaction mixture. Zero-time controls were run to correct for nonenzymatic radioactivity. After two developments (18 cm) in *n*-propanol–ethyl acetate–water (70:20:10), the chromatogram was dried, compounds were scraped from the plate, and radioactivity was determined in 5 ml of Aquasol. Radioactive glucans did not migrate from the origin of the chromatogram. The $R_{sucrose}$ values (sucrose = 1.00) determined for reference compounds were: raffinose, 0.24; isomaltose, 0.33; maltose, 0.71; glucose, 1.28; and fructose, 1.37.

Other assays. Protein was determined by the method of Lowry et al. (28), carbohydrate was determined by the anthrone method (38), and reducing sugars were determined by the ferricyanide method (41). Glucose formation was measured enzymatically by means of Worthington glucostat special reagents (Worthington Biochemicals Corp., Freehold, N.J.).

Dextranase experiments. Radioactive glucans were prepared from [*U-glucosyl*-^{14}C]sucrose by using various *S. mutans* 6715 glucosyltransferase fractions. The glucans were precipitated with 75% ethanol, solubilized in 1 N NaOH, and neutralized with acetic acid. These products were then tested as substrates for a dextranase from *Penicillium funiculosum* (5,000 U/mg [dry weight]; Merck Sharpe & Dohme Research Laboratories). Enzyme units are those described by Chaiet et al. (5). This exodextranase preparation is specific for α-1,6 linkages and will not hydrolyze α-1,3 bonds (T. Stoudt, Merck Sharpe & Dohme Research Laboratories, personal communication). The glucans were incubated under toluene with dextranase (625 U/290 μl) for 18 h at 37°C in 75 mM potassium phosphate buffer, pH 6.0. Duplicate samples were treated in the same manner in the absence of dextranase to determine the effect of 1 N NaOH treatment on the glucan products. Glucans were then precipitated by the addition of 3 volumes of cold absolute ethanol. Radioactive products soluble in the 75% ethanol supernatant fluid after centrifugation at 35,000 × *g* were measured by counting portions in a liquid scintillation counter, using 5 ml of Aquasol. The results were compared with the radioactivity in controls containing the same amounts of glucans solubilized in 1 N NaOH and immediately counted in the scintillation counter.

In other experiments, commercial *Leuconostoc mesenteroides* B512 dextrans (Sigma Chemical Co.,

St. Louis, Mo.) were incubated with the fungal dextranase (1,000 U/mg of dextran) for 2 h at 37°C in 10 mM potassium phosphate buffer, pH 6.0. The samples were then placed in boiling water for 3 to 5 min to terminate the reaction and were subsequently clarified by centrifugation at 35,000 × g. The 35,000 × g supernatant fluid was tested for its effect on the synthesis of water-soluble and water-insoluble glucans by S. mutans 6715 glucosyltransferases.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Chung et al. (8). Samples were incubated at 37°C for 1 h with a solution of 50 mM sodium phosphate (pH 7.1), 0.10% SDS, 5 M urea, and 5% 2-mercaptoethanol; gels (5%) were polymerized with a solution of 0.05% SDS, 6 M urea, and 4 mM ethylenediaminetetraacetate. Molecular weight standards were those of Chung et al. (8), prepared by treating purified transglutaminase with a chemical cross-linking reagent, dimethylsuberimidate. Duplicate gels containing approximately 50 μg of glucosyltransferase protein were stained with Coomassie brilliant blue to detect protein (7) and by the periodic acid-Schiff method to detect carbohydrate (44).

Gel filtration. Molecular weights of the various glucosyltransferases were estimated by gel filtration on a Bio-Gel A-0.5 column (34 by 2.5 cm; Bio-Rad Laboratories) equilibrated with 10 mM imidazole hydrochloride (pH 6.5) containing 100 mM KCl and 0.03% NaN_3. The column was calibrated with ribonuclease, chymotrypsinogen, ovalbumin, and aldolase (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.); plasma protransglutaminase (gift from S. I. Chung); and glutamine synthetase (9). Two-milliliter fractions were collected, and protein was determined by measuring the absorbance at 230 nm. Fractions were assayed for glucosyltransferase activity by measuring the incorporation of [*U-glucosyl*-^{14}C]sucrose into total glucans and into water-soluble glucans as described previously.

Purification of glucosyltransferases. All glucosyltransferase purification steps were carried out at 4°C unless otherwise specified.

Step 1: Culture supernatant fluid. Cells were harvested from the complex medium after overnight growth with a DeLaval separator (DeLaval Separator Co., Poughkeepsie, N.Y.), and the culture supernatant fluid was then made 0.02% with respect to NaN_3 and cooled to 4°C. The final volume was 20 liters.

Step 2: Concentration and ultrafiltration. The culture supernatant fluid was concentrated to approximately 145 ml with PM 10 Diaflo ultrafiltration membranes (Amicon Corp., Lexington, Mass.) in an Amicon ultrafiltration cell. The concentrated sample was clarified by centrifugation at 35,000 × g and subjected to continuous diafiltration in the same apparatus with 5 volumes of imidazole buffer (10 mM imidazole- hydrochloride [pH 6.5] containing 0.02% NaN_3) to remove molecules that could pass through the PM 10 membrane.

Step 3: XM 100A ultrafiltration. Ultrafiltration of the step 2 retentate (145 ml) was carried out by continuous diafiltration with 20 volumes of the imidazole buffer in a stirred Amicon cell containing an XM 100A Diaflo ultrafiltration membrane (Amicon) and then concentrated to 101 ml in the same system.

Step 4: XM 300 ultrafiltration. Imidazole buffer was added to the step 3 retentate to a final volume of 153 ml, and ultrafiltration of this preparation was carried out as described in step 3, except that an XM 300 Diaflo ultrafiltration membrane (Amicon) was used.

Step 5: Ethanol precipitation. The step 4 retentate (153 ml) was clarified by centrifugation at 35,000 × g. The supernatant solution was treated with 3 volumes of cold absolute ethanol (−20°C), and the precipitate was dissolved in 1 volume of buffer. The precipitation was repeated to yield the unfractionated enzyme (UFE) preparation.

The step 5 preparation (UFE) was resolved into two major, catalytically active components by fractional ethanol precipitation. To 5-ml volumes of the step 5 preparation, cold absolute ethanol (−20°C) was added to 33.3%, and the precipitate was discarded. Cold ethanol was further added to 50%, and the precipitate was collected and redissolved in 2 ml of 10 mM imidazole hydrochloride (pH 6.5) containing 0.02% NaN_3. This fraction produced a water-insoluble product from sucrose (see Results) and was designated as insoluble product enzyme (IPE). Ethanol was added to the 33.3 to 50% supernatant fraction to 60%, and the precipitate was collected and redissolved as described above. This fraction produced a water-soluble product from sucrose (see Results) and was designated as soluble product enzyme (SPE).

The enzyme preparations were stored at 4°C in 10 mM imidazole hydrochloride buffer (pH 6.5) containing 0.02% NaN_3. After approximately 18 months of storage in this manner, the UFE preparation retained 67%, the SPE fraction retained approximately 51%, and the IPE fraction retained 66% of their original enzyme activity (based on total glucan produced).

RESULTS

Purification and resolution of the glucosyltransferase. The purification method was selected to keep the loss of glucosyltransferase activity to a minimum. A summary of the results is given in Table 1. The procedure yielded a 239-fold-purified preparation (based upon total glucan synthesized from sucrose), with an 87% recovery of enzyme units and a specific activity of 9.56 U/mg of protein. The step 5 preparation had a carbohydrate/protein ratio of 3.33, which represents a carbohydrate enrichment of about twofold over the dialyzed PM 10 concentrate (step 2).

That the step 5 preparation (UFE) was likely an enzyme complex was suggested by the fact that it could be resolved into two catalytically active components by ethanol fractionation (see Materials and Methods for details). One fraction, IPE, precipitated between 33.3 and 50% ethanol and produced a water-insoluble product from sucrose (Table 2). A second fraction, SPE, precipitated between 50 and 60% ethanol and pro-

TABLE 1. *Purification of the glucosyltransferases of S. mutans 6715*

Step	Total units[a]	Protein (mg/ml)	Sp act (U/mg of protein)	Purification (fold)	Recovery (%)	mg of carbohydrate/mg of protein
1. Culture supernatant	3,010	4.1	0.04		100	
2. PM 10 concentration and ultrafiltration	2,944	10.5	1.93	48.3	98	1.88
3. XM 100A ultrafiltration	2,872	4.90	5.80	145.0	95	2.30
4. XM 300 ultrafiltration	2,795	3.10	5.89	147.3	93	2.23
5. Ethanol precipitation (UFE)	2,632	1.80	9.56	239.0	87	3.33

[a] Assay based on total glucan produced from [*U-glucosyl*-^{14}C]sucrose as described in the text.

TABLE 2. *Comparison of the step 5 preparation (UFE) with its two catalytically active components*

Enzyme[a] prepn	Total[b] units	Sp act (U/mg of protein)	Glucan synthesis[c]		mg of carbohydrate/mg of protein
			Insoluble	Soluble	
UFE	2,632	9.56	2.77	0.06	3.33
IPE	644.9	3.60	0.79	0	3.78
SPE	551.6	10.60	0.15	3.01	1.65

[a] See text for description of enzyme preparation.
[b] Assay based on total glucan produced as described in the text.
[c] Micromoles of [*U-glucosyl*-^{14}C]sucrose incorporated into water-insoluble or water-soluble glucans in 30 min per 10 μg of protein.

duced a water-soluble product from sucrose (Table 2). These two fractions differed not only in the carbohydrate polymer produced, but also with respect to their relative specific activities and their respective carbohydrate/protein ratios.

Products of the glucosyltransferase preparations. Fraction UFE synthesized primarily water-insoluble glucans when incubated for 60 min under conditions of sucrose saturation (Fig. 1). Double-reciprocal plots (11) of kinetic data obtained at various sucrose concentrations showed that the UFE preparation is saturated at 35 to 40 mM sucrose and has a K_m for sucrose of approximately 5 mM (data not shown). Whereas Fig. 1 shows that the incorporation of the glucosyl moiety of sucrose into insoluble glucans is linear for 60 min, neither water-soluble nor insoluble polysaccharides were produced from the fructosyl moiety of sucrose under the same assay conditions. It should be noted, however, that after extended incubation the UFE preparation did produce some water-soluble glucan. A thin-layer chromatographic analysis of the products formed after incubating the UFE preparation for 24 h with sucrose showed that, of 53.7 μmol of glucose polymers synthesized, 11.1 μmol was water soluble. Another product of the 24-h assay isolated by this procedure represented 4.1% of the total radioactivity recovered and had a mobility expected for a trisaccharide, but was not further characterized. This latter compound was not detected among the products of the 60-min assay.

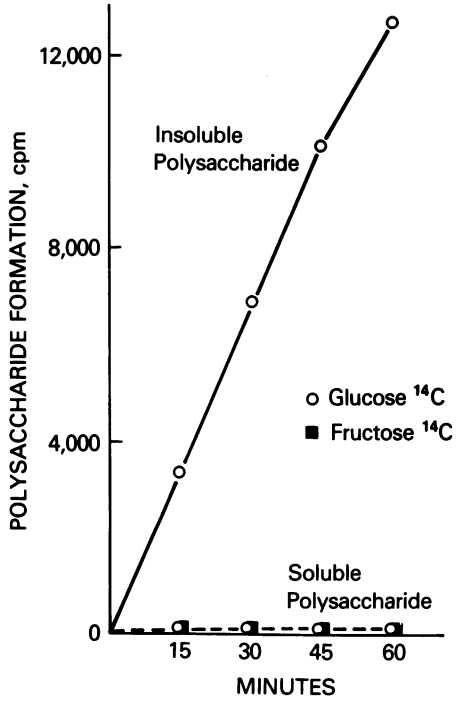

FIG. 1. *Incorporation of radioactivity from [glucosyl-*^{14}*C]- and [fructosyl-*^{14}*C]sucrose into water-insoluble and water-soluble polysaccharides by the UFE preparation. UFE (Table 1; 18 μg/ml) was assayed for incorporation of the glucosyl-*^{14}*C-labeled moiety* (○) *or the fructosyl-*^{14}*C-labeled* (■) *moiety of sucrose into polysaccharides.*

Free glucose was also consistently found as a reaction product with the UFE preparation. At 60 min the glucose produced was approximately 14% of the total reducing sugars found (Fig. 2). The free glucose apparently did not arise as a result of a contaminating invertase that could hydrolyze raffinose (30), because the UFE preparation was unable to release fructose from raffinose under conditions in which it converted more than 20% of an equivalent amount of sucrose (20 mM) into glucans and fructose.

The two fractions resolved from the UFE preparation by ethanol treatment catalyzed the synthesis of different products from sucrose. The IPE fraction, like the UFE preparation, made water-insoluble glucans, whereas the SPE fraction made primarily soluble glucans (Table 2).

Dextranase hydrolysis of the glucans synthesized. The glucan products synthesized by the various enzyme fractions exhibited differential susceptibility to hydrolysis by fungal dextranase. The initial treatment of all glucan samples with 1 N NaOH (to dissolve insoluble glucans) followed by neutralization converted only a small amount of the glucans to products soluble in 75% ethanol (3.1 to 5.4%) (Table 3). However, subsequent treatment of the dissolved glucans with dextranase yielded products with significant differences in ethanol solubility. The glucans produced by the SPE preparation were rendered completely soluble by dextranase, whereas only 65% of the IPE glucans became soluble after this treatment. The glucans formed by the UFE preparation (which contains both the IPE and SPE components) were affected by dextranase to an intermediate degree.

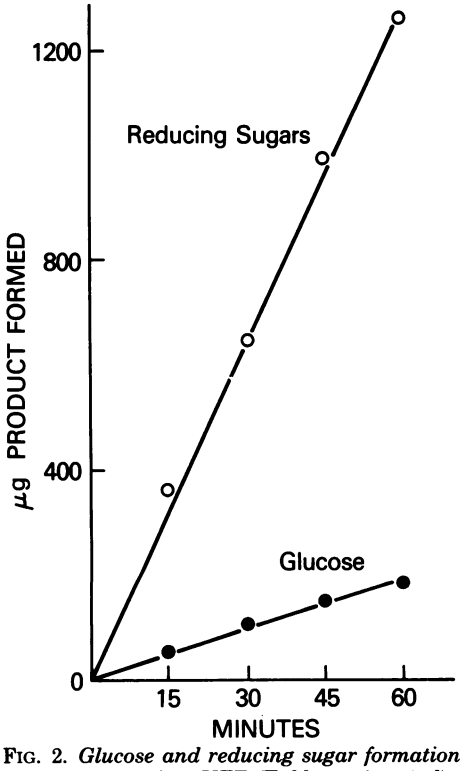

FIG. 2. *Glucose and reducing sugar formation by the UFE preparation. UFE (Table 1; 18 μg/ml) was assayed for formation of glucose (●) and reducing sugars (○) as described in the text.*

TABLE 3. *Effect of dextranase on ^{14}C-labeled glucans synthesized by various glucosyltransferase preparations of S. mutans 6715*[a]

Enzyme prepn	Major glucans synthesized	% ^{14}C-labeled products soluble in 75% ethanol[b]		
		Control	1 N NaOH treatment	Dextranase treatment
UFE	Water insoluble	0	3.3	78
IPE	Water insoluble	0	5.4	65
SPE	Water soluble	0	3.1	100

[a] *U-glucosyl*-^{14}C-labeled glucans were isolated from a 60-min incubation of the indicated enzyme preparation with 50 mM [*U-glucosyl*-^{14}C]sucrose under standard assay conditions and treated as described in the text.

[b] Results are expressed as a percentage of ^{14}C-labeled products remaining in the 75% ethanol supernatant after centrifugation at 35,000 × *g* for 20 min.

Interaction of IPE and SPE components. An interaction of the IPE and SPE components in the formation of glucans by the UFE preparation is suggested by the results shown in Fig. 3, which compares the kinetics of insoluble glucan formation by these enzyme preparations using both turbidimetric and radioactive assays. Under assay conditions in which the UFE and IPE preparations synthesized insoluble glucans and the SPE produced primarily soluble glucans, little change in turbidity over 60 min occurred in the SPE preparation (absorbance equals 0.08 at 60 min). In contrast, both the IPE and UFE preparations showed significant increases (absorbances at 60 min equal 0.83 and 0.77, respectively). The linear rate of water-insoluble glucan formation was slightly greater for UFE than for IPE (absorbance changes of 0.016 versus 0.015/min). The initial lag in the time curve was also significantly greater for the UFE preparation (12 versus 4 min for IPE). The incorporation of the labeled glucosyl moiety of sucrose into glucan products at 60 min was 4,433 cpm for IPE, 7,216 cpm for UFE, and 23,955 cpm for SPE. A comparison of the results obtained for the UFE and IPE preparations, i.e., a longer initial lag phase and a higher incorporation of radioactive glucose counts per absorbance unit at 340 nm for the UFE preparation, suggests

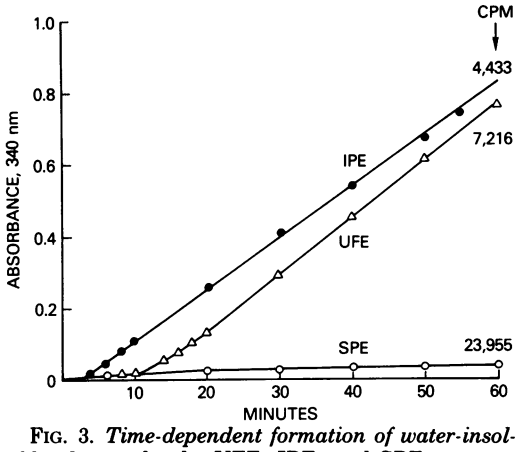

FIG. 3. *Time-dependent formation of water-insoluble glucans by the UFE, IPE, and SPE preparations. Enzyme fractions (UFE, 3.6 μg; IPE, 5.9 μg; and SPE, 8.5 μg) were incubated at 30°C in a final volume of 1.0 ml under standard assay conditions. Insoluble glucan formation was measured as the change in absorbance at 340 nm. Total glucosyl-^{14}C-labeled glucans were determined in a 100-μl portion at 60 min of incubation as described in the text.*

that, during initial glucan synthesis, water-soluble products, probably made by the SPE component, may be incorporated into insoluble polymers in the UFE preparation. Additional evidence for this conclusion came from reconstitution experiments. When various proportions of IPE and SPE were mixed, more total glucan (up to approximately twofold more) was consistently produced than the additive amounts produced by each fraction assayed individually (data not shown). Kuramitsu (27) has also reported that ^{14}C-labeled soluble glucans produced by a glucosyltransferase of *S. mutans* GS-5 could to a "limited extent" be incorporated into water-insoluble glucans in the presence of sucrose by an enzyme fraction that produced 56% insoluble glucans and 44% soluble glucans.

SDS-polyacrylamide gel electrophoresis. When the step 4 enzyme preparation (Table 1) was subjected to SDS-urea polyacrylamide gel electrophoresis and the resulting gel was stained with Coomassie brilliant blue, several protein bands were detected (Fig. 4, middle gel). The molecular weights estimated for these bands were between 100,000 and 212,000, with the major band having an apparent molecular weight of 175,000. A duplicate gel stained for glycoprotein (Fig. 4, right-hand gel) shows that all of the bands containing protein also developed a positive periodic acid-Schiff stain, indicating that a carbohydrate moiety is tightly bound to each.

Molecular weights by gel filtration. When subjected to gel filtration on an agarose A-0.5 column, the UFE preparation exhibited a protein peak with an estimated molecular weight of 150,000 [Fig. 5, GT(major)]. The fraction under this peak synthesized only insoluble glucans in a 20-h assay. Fractions corresponding to molecular weights greater than 150,000 synthesized increasing amounts of water-soluble glucans; maximum recovery of these polymers (34% of the total glucans isolated in the fraction) was reached in a fraction with an elution volume corresponding to a molecular weight of 220,000 [Fig. 5, GT(soluble glucan)]. However, on the same column the SPE fraction, which synthe-

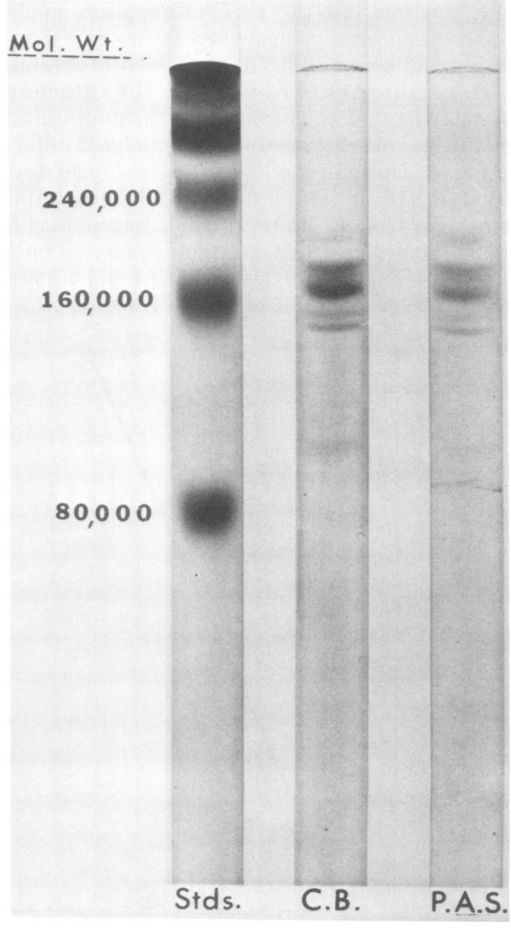

FIG. 4. *SDS-polyacrylamide gel electrophoresis patterns of glucosyltransferases. From left to right, the first gel (Stds) contained molecular weight standards (see text) and was stained for protein with Coomassie brilliant blue. The second (C.B.) and the third (P.A.S.) gels each contained 50 μg of enzyme preparation purified through the XM 300 step (Table 1) and were stained, as described in the text, with Coomassie brilliant blue for protein and with periodic acid-Schiff for carbohydrate, respectively.*

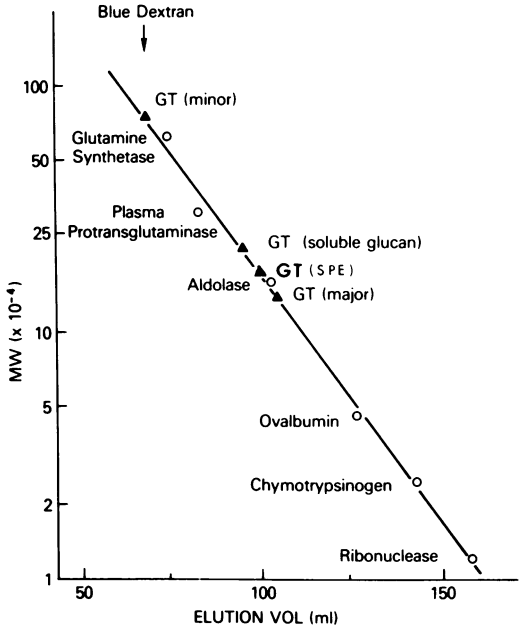

FIG. 5. *Estimation of molecular weights of glucosyltransferases by gel filtration on Bio-Gel A-0.5. Symbols: ○, standard proteins; ▲, glucosyltransferases. See text for abbreviations.*

sized water-soluble glucans and formed one major protein band upon polyacrylamide gel electrophoresis, exhibited a protein peak with an estimated molecular weight of 175,000 [Fig. 5, GT(SPE)].

A minor protein peak containing glucosyltransferase activity [Fig. 5, GT(minor), molecular weight >500,000] and representing less than 5% of the total enzyme recovered consistently appeared in the column void volume from both the UFE and SPE preparations.

Effect of preformed glucans on glucosyltransferase activity. Our observation that *S. mutans* 6715 soluble glucan products inhibited synthesis of insoluble glucans (unpublished data) and those reported by Gibbons and Keyes (21) on a similar inhibition by *Leuconostoc* dextrans, led to a study of the effects of various glucans on product synthesis by the UFE preparation. Soluble glucans synthesized by the SPE preparation and three commercial *Leuconostoc* dextrans with average molecular weights of 20,000 to 2,000,000 cause a pronounced inhibition of insoluble glucan synthesis by the UFE preparation (Table 4). The *Leuconostoc* dextrans also showed an apparent stimulation of soluble glucan synthesis by UFE that was not seen with the soluble glucans produced by the SPE preparation. Pretreatment of the dextrans with fungal dextranase gave hydrolytic products that had no effect on glucan synthesis by the UFE preparation.

In contrast, a soluble glucan preparation isolated after a long incubation (20 h) of a *S. mutans* 6715 culture supernatant with sucrose (soluble glucan-CS) caused a marked stimulation of insoluble glucan synthesis (Table 4). Concomitant with this effect was a decrease in absorbance due to water-soluble polysaccharides.

DISCUSSION

The purification procedure shown in Table 1, unlike those previously reported for *S. mutans* glucosyltransferases (6, 15, 26, 27, 33), does not utilize hydroxyapatite columns or gel filtration.

TABLE 4. *Effect of added glucans on glucosyltransferase activity*[a]

Addition[b]	μg	Absorbance at 340 nm[c]	
		Insoluble glucans	Soluble glucans
None	0	0.128	0.004
Soluble glucans-SPE	40	0.073	0.002
	200	0.033	0.006
Dextran 15, avg mol wt = 2×10^4	100	0.102	0.136
	200	0.021	0.256
Dextran 200c, avg mol wt = 2×10^5	100	0.006	0.191
	200	0.009	0.336
Dextran 2000, avg mol wt = 2×10^6	100	0.069	0.110
	200	0.015	0.185
Dextranase-treated Dextran 15	100	0.144	0.006
	200	0.123	0.007
Dextranase-treated Dextran 200c	100	0.136	0.010
	200	0.144	0.008
Dextranase-treated Dextran 2000	100	0.129	0.009
	200	0.139	0.006
Soluble glucans-CS	100	0.199	−0.034
	200	0.509	−0.066

[a] The UFE preparation (1.8 μg/ml) was preincubated for 15 min at 37°C with the glucans and assayed for new glucan synthesis after 2 h at 37°C with 40 mM sucrose under otherwise standard turbidimetric assay conditions.

[b] Dextrans (Sigma Chemical Co.) were produced by *L. mesenteroides* B512. Soluble glucans-SPE were synthesized by the SPE preparation after 4 h of incubation with sucrose under standard assay conditions. Soluble glucans-CS were isolated from a culture supernatant of strain 6715 (grown on 0.2% glucose) with 3 volumes of absolute ethanol after a 20-h incubation at 37°C with 5% sucrose and prior removal of water-insoluble glucans. Dextranase treatment of dextrans is described in the text. All glucan additions were placed in a boiling-water bath for 3 to 5 min before being tested to inactivate any enzyme activity that might be associated with them.

[c] Absorbance was corrected for turbidity by a blank without enzyme. Zero-time control with only enzyme showed a negligible absorbance. Soluble glucans were determined as described in the text.

The latter procedures were investigated as early purification steps and were found to lead to high-molecular-weight aggregates of the enzyme and a significant loss of activity (unpublished data). Instead, a sequence of steps involving concentration, ultrafiltration, and diafiltration with Amicon membranes followed by ethanol precipitation has led to a glucosyltransferase preparation (UFE) with an 87% yield of enzyme units and a relatively high specific activity of 9.56 U/mg of protein. The specific activity determined at 30°C is comparable to or higher than those determined at 37°C for glucosyltransferases by other investigators (6, 26, 27, 33).

The UFE preparation did not contain either fructosyltransferase or invertase activities under the assay conditions employed, but free glucose was consistently found as a reaction product (Fig. 2). Because glucose formation was previously shown to be directly associated with each of several different glucosyltransferase bands separated by polyacrylamide gel electrophoresis (10), hydrolysis of sucrose by the glucosyltransferases themselves seems likely. A similar conclusion has been reported for glucose formation by glucosyltransferases from *S. mutans* HS-6 (15) and *L. mesenteroides* (23).

As observed with other strains of *S. mutans* (15, 16, 27, 33), the glucosyltransferase of strain 6715 could be resolved into fractions that synthesize either water-soluble (SPE) or water-insoluble glucans (IPE), with one significant difference. In the present study the enzyme preparations synthesizing insoluble and soluble glucans were found to have molecular weights between 150,000 and 175,000 (Fig. 5). Although a molecular weight of 94,000 was reported by Chludzinski et al. (6) for a glucosyltransferase from strain 6715, this value might be a low estimate because the enzyme was eluted near the void volume of the Bio-Gel P 150 column, and the largest standard protein used in determining this value had a molecular weight of 79,000. Our results (Fig. 4 and 5) and similar molecular weights determined by SDS-polyacrylamide gel electrophoresis for several different glucosyltransferase preparations (10, 15, 27) suggest a significant degree of structural stability for these functional forms of the enzymes. However, treatment of either the IPE or SPE fraction with SDS at elevated temperature results in a multiplicity of protein bands of decreasing molecular size after polyacrylamide gel electrophoresis (10). Because of these results and recent reports of a molecular weight of 45,000 determined by SDS-polyacrylamide gel electrophoresis for the soluble glucan-synthesizing enzyme of *S. mutans* GS-5 (27) and a molecular weight of 40,000 determined by gel filtration for a glucosyltransferase from *S. mutans* 6715 (19), it appears that our functional enzymes may still exist as aggregated or oligomeric forms. The relatively high stability of these forms may be due to the tightly bound carbohydrate associated with the enzymes (10) (Fig. 4).

Data presented in this study suggest that the IPE and SPE components of the UFE preparation may interact to synthesize mixed insoluble glucans different than those made by the IPE preparation alone (Fig. 3). The glucans made by UFE are hydrolyzed by fungal dextranase, which has a specificity for α-1,6 linkages, to a greater degree than those made by the IPE preparation (Table 3). The SPE glucans that are water-soluble are rendered completely soluble in 75% ethanol after treatment with dextranase. These data suggest that the SPE glucan contains primarily α-1,6 linkages, that the IPE products contain a high percentage of non-α-1,6, probably α-1,3 bonds (4, 12, 13), and that the UFE product contains intermediate amounts of both types of linkages.

The fact that several protein bands associated with insoluble glucan synthesis have been resolved from the IPE preparation by polyacrylamide gel electrophoresis (10) suggests multiple forms of the glucosyltransferase. Other experiments showed that these glucan-synthesizing enzymes differ in net charge or molecular size or both (10). Guggenheim and Newbrun (26) also isolated several glucosyltransferases with different isoelectric points from *S. mutans* OMZ-176, also a serotype *d* strain. In contrast, Chludzinski and co-workers (6) isolated only two glucosyltransferases by polyacrylamide gel electrophoresis from *S. mutans* 6715. These investigators, however, employed a purification procedure entirely different from the one utilized in the present and previous studies (10). Moreover, their use of Trypticase soy broth as a growth medium, which apparently contains some sucrose and/or dextran (18, 40), may have resulted in a large amount of the extracellular glucosyltransferase becoming cell associated. Spinell and Gibbons (40) found that with Trypticase soy broth 40% of the total glucosyltransferase activity of strain 6715 was cell associated.

The presence of multimolecular forms of glucosyltransferase (10) may also be due to different amounts of carbohydrate associated with the enzymes (Table 2; Fig. 4). This has been shown to be the case for certain glycoenzymes (36). Carbohydrate has been observed in purified glucosyltransferase preparations by several other investigators (18, 27, 33). We reported previously that a carbohydrate moiety of the glucosyltrans-

ferases from *S. mutans* 6715 could not be removed by boiling in SDS or by extensive treatment with dextranase (10) and suggested that it may be involved in a glycoprotein-like structure rather than being present as a noncovalently bound moiety.

Gibbons and Keyes (21) have shown the inhibitory effects of high-molecular-weight dextrans on the synthesis of water-insoluble glucans by *S. mutans*. A similar effect is seen on insoluble glucan synthesis by the UFE preparation (Table 4). The concomitant increase in water-soluble glucan synthesis (Table 4) agrees with previous reports on the stimulation of *S. mutants* glucan synthesis by dextrans (15, 16, 18, 27, 29). Germaine et al. (18) and Kuramitsu (27) isolated glucosyltransferases that actually show a requirement for dextran for catalytic activity. In contrast to the effects observed with commercial *Leuconostoc* dextrans, a soluble glucan produced by the SPE fraction had no effect on the synthesis of water-soluble glucan by the UFE preparation, although it inhibited the synthesis of water-insoluble glucan by the same preparation (Table 4). The reason for the inability of the SPE glucan to act as an acceptor molecule for soluble glucan synthesis is unknown and awaits further studies. Dextranase treatment of the *Leuconostoc* dextrans abolished both the inhibitory effect on insoluble glucan synthesis and the stimulation of soluble glucan formation.

The stimulation of insoluble glucan formation by a soluble glucan product obtained from *S. mutans* 6715 culture media after extended incubation with sucrose (Table 4) was unexpected in light of the results obtained with *Leuconostoc* dextrans. It is unlikely that glucosyltransferase activity that appeared to remain with the glucan preparation after boiling for 3 to 5 min could account for the increased amount of insoluble polysaccharides synthesized. However, if an endohydrolytic dextranase activity (25, 42, 43) was also present subsequent to boiling, the glucans might have been hydrolyzed to low-molecular-weight polysaccharides or oligosaccharides that could act as efficient acceptors for insoluble glucan synthesis in the presence of glucosyltransferase (18, 34). This appears possible because of the accumulation of a significant amount of a low-molecular weight, glucosyl-labeled compound after a 24-h incubation but not after a 1-h incubation of the UFE preparation with radioactive sucrose. The results show that soluble glucan preparations may either inhibit or stimulate insoluble polysaccharide synthesis. It appears probable, therefore, that interactions of the *S. mutans* glucan-synthesizing enzyme system with glucans present in dental plaque, synthesized by *S. mutans* or other oral bacteria, could greatly influence the synthesis of adherent glucans by these bacteria.

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